Phytochemistry, 1974, Vol. 13, pp. 1099–1105. Pergamon Press. Printed in England.

STEROL ESTERS OF *PHYCOMYCES BLAKESLEEANUS*

E. IAN MERCER and KIM BARTLETT*

Department of Biochemistry and Agricultural Biochemistry, University College of Wales, Aberystwyth SY23 3DD, Wales

(*Received* 8 *August* 1973)

Key Word Index—*Phycomyces blakesleeanus*; Muroraceae; sterol esters; sterols; fatty acids; triglycerides; phospholipids.

Abstract—The sterol esters of *Phycomyces blakesleeanus* are based on ergosterol, episterol, ergosta-7-en-3β-ol, ergosta-7,22-dien-3β-ol, cholesterol, lanosterol and 24-methylenelanost-8-en-3β-ol. The fatty acid composition of the esters differs significantly from those of the triglyceride and phospholipid fractions of this fungus.

INTRODUCTION

THE COMPOSITION of the sterol esters of higher plants has been the subject of several investigations;[1–3] in addition, studies of the sterol esters in relation to the neutral lipid content of higher plants have been made.[4–10] However, the composition of the sterol esters of the fungi has received little attention. Ergosterol palmitate has been shown to occur in *Penicillium* spp[11,12] and *Aspergillus fumigatus*[13] whilst ergosterol stearate has been isolated from *Lactarius deliciosus*.[14] A comparison of the fatty acids esterified to sterols and polyisoprenoid alcohols in *A. fumigatus* has been reported,[15] as has the fatty acid composition of the sterol esters of *Saccharomyces cerevisiae* grown under varied cultural conditions.[16] The presence of sterol esters in *Phycomyces blakesleeanus* was first demonstrated by Jacks[17] and subsequently confirmed.[18] Although the total sterol composition of this organism has been investigated,[18,19] an analysis of the sterol esters has not been carried

* Present address: Department of Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, London W.12.

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out. Furthermore, to our knowledge, no analyses of the sterol moieties of the sterol esters of any fungus have been reported. In this study we have analysed the sterol and fatty acid components of the sterol esters of *P. blakesleeanus* and have compared the fatty acid composition of the sterol esters with that of the triglycerides and two phospholipid fractions.

RESULTS

Characterization of the sterol moieties of the sterol esters

The lipid (4·095 g) was extracted from the mycelium from 14 1·5 l., 60 hr *P. blakesleeanus* cultures. The majority of the unesterified sterol was removed from the lipid by crystallization from 10 ml petrol. The residual lipid was chromatographed on a 150 g alumina column which was developed with 4% (v/v) Et_2O in petrol. (E/P) so as to elute the sterol esters. The 4% E/P fraction (0·999 g) was shown to be free of unesterified sterols by TLC (systems 1 and 2) and then saponified. The resulting unsaponfiable material (185·3 mg) was separated by chromatography on a 20 g alumina column into the following fractions (1) 3% E/P (16·0 mg), (2) 6% E/P (5·4 mg), (3) 9% E/P (12·6 mg), (4) 12% E/P (12·9 mg), (5) 20% E/P (139·4 mg). TLC (system 1) showed that all the 4-demethylsterols were present in fraction 5 whilst all the 4,4-dimethylsterols were in fractions 3 and 4.

The 4-demethylsterols (fraction 5) were purified by TLC (system 1) and then separated into three zones (1–3) by argentation TLC. Zone 1 (R_f 0·20) cochromatographed with ergosterol and had a UV spectrum in EtOH characteristic of $\Delta^{5,7}$-sterols[20] (maxima at 272, 282 and 293 nm; shoulder at 262 nm). The IR spectrum was identical with that of ergosterol; strong absorption at 800–834 cm^{-1}, characteristic of $\Delta^{5,7}$ unsaturation,[21] and at 970 cm^{-1}, characteristic of *Trans*-Δ^{22} unsaturation[22] were observed. However, GLC on 5% QF-1 separated zone 1 into a major component (RR_t* 3·27) which constituted 90% of the zone 1 sterol and cochromatographed with ergosterol and a minor component (RR_t 3·77). The minor component could not be removed from the major component, which appeared to be ergosterol, by repeated adsorption and argentation TLC. GC–MS of zone 1 (Fig. 1a) produced three peaks, A–C. The MS of peak A showed that it was not a sterol. The MS (Table 1) of the major sterol, peak B, had a molecular ion at *m/e* 396 suggesting a C_{28} sterol with three double bonds. Ions *d*, *e*, *f*, *i* and *j* at *m/e* values of 269, 253, 251, 299 and 211 respectively show that two of these double bonds are nuclear, whilst ion *t* at *m/e* 337 identifies them as a $\Delta^{5,7}$ conjugated system.[23,24] Ions *d*, *e and f* show that the side chain has an extra methyl group, presumably located at C-24. Ions *p* and *q* at *m/e* values of 298 and 283 respectively show the presence of a Δ^{22} double bond. The MS therefore shows that sterol B is ergosterol. The MS of peak C (Table 1) was consistent with its being principally composed of ergosta-5,7 24(28)-trien-3β-ol.[25]

Zone 2 (R_f 0·35) was separated by GLC on both 5% QF-1 and 3% OV-1 into two components, a major one (RR_t on QF-1 and OV-1, 3·82 and 2·76 respectively) which cochromatographed with episterol and a minor one (RR_ts on 5% QF-1 and 3% OV-1, 3·12 and 2·54 respectively).

* Retention time relative to cholestane.

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The IR spectrum of zone 2 strongly resembled that of episterol; strong absorption at 890 and 1650 cm^{-1} being consistent with the presence of a terminal methylene group.[18] GC–MS of zone 2 (Fig. 1b) produced the major (E) and minor (D) peaks, but insufficiently well separated to get an adequate MS of peak D. The mass spectrum of peak E (Table 1) had a molecular ion at *m/e* 398 suggesting a C_{28} sterol with two double bonds. Ions *d*, *e*, *f*, *g*, *h*, *i* and *j* at *m/e* values of 271, 255, 253, 246, 228, 231 and 213 respectively show that one of these double bonds is nuclear; moreover the base peak, ion *d*, at *m/e* 271 favours Δ^7 unsaturation.[26,27] Ions *k* and *l* at *m/e* values of 314 and 299 respectively indicate that there is a $\Delta^{24(28)}$ double bond. The MS therefore shows that sterol E is episterol.

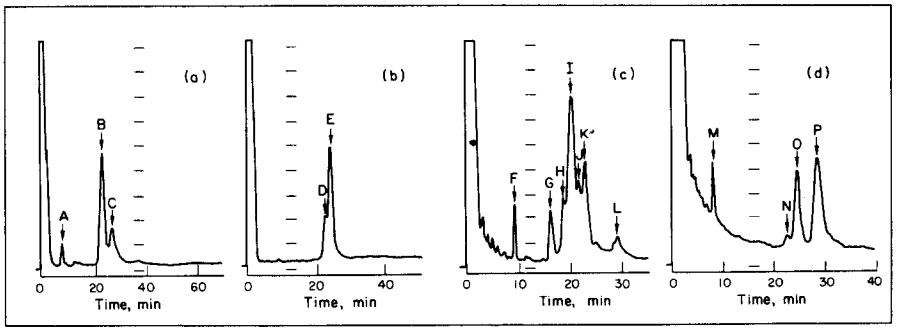

FIG. 1. GC—MS OF STEROLS DERIVED FROM THE STEROL ESTERS OF *Phycomyces blakesleeanus*. A—4-demethyl sterols from zone 1 of argentation TLC; B—4-demethyl sterols from zone 2 of argentation TLC; C—4-demethyl sterols from zone 3 of argentation TLC; D—4,4-dimethyl sterols. The GC column was 1% JXR at 250° and the carrier gas was helium. MS were taken at the points indicated by the arrows.

Zone 3 (R_f 0·44, 3·1 mg) was separated by GLC on 5% QF-1 into five components RR_ts 2·82, 3·22, 3·57, 3·87 and 4·09. GC–MS of this zone (Fig. 1c) produced seven peaks (F–L) of which five (G–K), judging from their MS, were sterols and presumably corresponded with the GLC peaks seen on 5% QF-1. Peaks H and J were too contaminated to produce MS of any diagnostic value. The MS of peak G had a molecular ion at *m/e* 386 suggesting a C_{27} sterol with one double bond. Ions *d*, *e*, *f*, *h*, *i* and *j* at *m/e* values of 271, 255, 253, 228, 231 and 213 respectively show that the double bond is nuclear. Ions *u*, *v* and *x* at *m/e* values of 301, 275 and 247 strongly suggest[27,28] that the position of the double bond is Δ^5. This, taken with the fact that the retention time of this sterol on 3% OV-1 corresponded with that of authentic cholesterol, indicates that sterol G is cholesterol. The MS of peak I has a molecular ion at *m/e* 398 suggesting that it is a diunsaturated C_{28} sterol. Ions *d*, *e*, *f*, *g*, *h*, *i* and *j* at *m/e* values of 271, 255, 253, 246, 228, 231 and 213 respectively show that one of the double bonds is nuclear; moreover the base peak, ion *d*, at *m/e* 271 favours Δ^7 unsaturation.[26,27] The presence of ions *n* and *o* at *m/e* values of 355 and 247 respectively signified the loss of an isopropyl group from the side chain, a fragmentation characteristic of Δ^{22} unsaturation. This was confirmed by the presence of ions *p* and *q* at *m/e* values of 300 and 285 respectively. Sterol I is therefore ergosta-7,22-dien-3β-ol. The MS of peak K had a molecular ion at *m/e* 400 suggesting a monounsaturated C_{28} sterol. Ions *d*, *e*, *f*, *g*, *h*, *i* and *j* at *m/e* values 271, 255, 253, 246, 228, 231 and 213 respectively show

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that the double bond is nuclear and the prominent nature of ion *d* suggests that it is located at the Δ^7 position. Sterol K therefore appears to be ergosta-7-en-3β-ol.

The 4,4-dimethylsterols (16·9 mg) were isolated from the bulked fractions 3 and 4 by TLC (system 1) and then analysed by GLC (5% QF-1). Two major peaks were observed with RR_ts of 3·90 and 4·31. The 4,4-dimethylsterols were then further fractionated into three zones, X (R_f 0·44; 4·2 mg), Y (R_f 0·38; 7·8 mg) and Z (R_f 0·32; 2·1 mg) by argentation TLC. Zone X co-chromatographed on argentation TLC with lanosterol. It gave a single peak when subjected to GLC on 5% QF-1 (RR_t 3·90) and 3% OV-1 (RR_t 3·05). The IR spectrum of this material and lanosterol were almost identical; both exhibited the weak absorption at 824 cm^{-1} due to the C–H out-of-plane bending of the C-24 hydrogen seen in Δ^{24} sterols.[21] Zone Y also gave a single peak when subjected to GLC on 5% QF-1 (RR_t 4·31) and 3% OV-1 (RR_t 3·67). The IR spectrum of this material was similar to that of lanosterol except that there were no absorption peaks in the 780–850 cm^{-1} region, suggesting the absence of trisubstituted double bonds, and there were strong 890 cm^{-1} and medium 1650 cm^{-1} absorptions, suggesting the presence of a terminal methylene group. This sterol thus appeared to be 24-methylene-lanost-8-en-3β-ol. The sterol of zone Z had the same RR_ts on both 5% QF-1 and 3% OV-1 as had the sterol of zone Y. Its IR spectrum differed from that of the zone Y sterol by the lack of a strong absorption peak at 1021 cm^{-1}.

TABLE 1. IONIC SPECIES IN THE MS OF THE STEROL MOIETIES OF THE STEROL ESTERS OF *Phycomyces blakesleeanus* (Intensities of the ions are shown in parentheses)

		Sterol								
Ion	Fragmentation	B	C	F	G	I	K	N	O	P
M^+	Molecular Ion	396(34)	396(27)	398(23)	386(44)	398(50)	400(100)	412(100)	426(36)	440(43)
a	M^+-Me	381(6)	381(28)	383(22)	371(17)	383(19)	385(23)	397(37)	411(100)	425(100)
b	M^+-HOH	378(53)	378(87)	380(11)	368(100)	380(21)	382(32)	394(13)	408(22)	422(24)
c	M^+-[Me + HOH]	363(62)	363(58)	365(9)	353(32)	365(8)	367(13)	379(25)	393(94)	407(100)
d	M^+-[SC + 2H]	269(17)	269(15)	271(100)	271(11)	271(100)	271(69)	285(37)	313(3)	313(5)
e	M^+-[SC + HOH]	253(100)	253(100)	255(27)	255(32)	255(60)	255(94)	269(37)	297(6)	297(15)
f	M^+-[SC + HOH + 2H]	251(47)	251(73)	253(34)	253(5)	253(31)	253(27)	267(43)	295(4)	295(6)
g	M^+-[SC + 27]			246(9)	246(6)	246(29)	246(8)	260(19)		
h	M^+-[SC + 27 + HOH]			228(9)	228(11)	228(17)	228(12)	242(12)	270(3)	270(3)
i	M^+-[SC + 42]	229(4)	229(9)	231(16)	231(12)	231(17)	231(26)	235(25)	273(3)	273(4)
j	M^+-[SC + 42 + HOH]	211(28)	211(51)	213(24)	213(36)	213(23)	213(43)	227(47)	255(13)	255(23)
k	M^+-[84]			314(37)			314(8)	328(6)		356(8)
l	M^+-[84 + Me]			299(10)			299(4)	313(6)		341(13)
m	M^+-[84 + Me + HOH]							295(12)		323(17)
n	M^+-[43]					355(9)				
o	M^+-[43 + HOH]					347(6)				
p	M^+-[C-22→ + H]					300(21)				
q	M^+-[C-22→ + H + Me]					285(3)				
r	M^+-[SC + 56]								259(13)	259(25)
s	M^+-[SC + 56 + HOH]								241(18)	241(37)
t	M^+-[59]	337(14)	337(9)							
u	M^+-[67 + HOH]				301(12)					
v	M^+-[93 + HOH]				275(29)					
x	M^+-[121 + HOH]				247(63)					

SC = side chain: 27 = [C-16 + C-17 + 3 H]: 42 = [C-15 + C-16 + C-17 + 6 H]: 84 = [C-23–C-28] in 24-methylene side chains of sterols other than those with a 9,19 cyclopropane ring: 43 = [C-24–C-27] in Δ^{22} side chains: [C-22→ + H] = H plus C-22 to the end of SC (84, 98 and 112 in C_{27}, C_{28} and C_{29} sterols respectively); only occurs in presence of Δ^{22} unsaturation: 56 = [C-15 to C-17 + C-32 + 8 H]: 59 = [C-1 to C-3 + OH + 6 H] characteristic of $\Delta^{5,7}$-sterols: 67 = C_5H_7 from C-2 to C-6 or C-3 to C-7 in Δ^5-sterols: 93 = C_7H_9 from rings A and B, probably C-1 to C-7, in Δ^5-sterols: 121 = C_9H_{13} from rings A and B by cleavage of the C-7 to C-8 and C-9 to C-10 bonds.

GC–MS of the 4,4-dimethylsterols (Fig. 1d) produced two major peaks (O and P) and a minor peak (N) which were clearly sterols; peak M was not a sterol. The MS of peak N had a molecular ion at *m/e* 412 suggesting a C_{29} sterol with two double bonds. Ions

k and *l* at *m/e* values of 328 and 313 point to the presence of a 24-methylene group and consequently a nucleus with one double bond and an extra methyl group. The absence of ions *r* and *s* at *m/e* values of 231 and 213, characteristic of 14α-methyl sterols,[29] place the extra methyl group at C-4. The probable identity of sterol N is 4α-methyl-ergosta-8,24(28)-dien-3β-ol. The MS of peak O had a molecular ion at *m/e* 426 suggesting a C_{30} sterol with two double bonds. Ions *d, e, f, h, i* and *j* at *m/e* values of 313, 297, 295, 270, 273 and 255 show that the side chain has 8 carbons and one double bond (C_8H_{15}). The absence of ions *n* and *o* (*m/e* values of 383 and 365 respectively) show that the side chain double bond is not at Δ^{22}; this is consistent with its location at Δ^{24}. Ions *r* and *s* at *m/e* values of 259 and 241 respectively indicate the presence of a 14α-methyl group.[29] Sterol O is therefore lanosterol and is the sterol present in zone X of the argentation TLC. The mass spectrum of peak P has a molecular ion at *m/e* 440, 14 m.u. higher than that of sterol O. Ions *d, e, f, h, i* and *j* at *m/e* values of 313, 297, 295, 270, 273 and 255 showed that the extra 14 m.u. resided in the side chain. Ions *k* and *l* at *m/e* values of 356 and 341 respectively showed the presence of a 24-methylene group. Ions *r* and *s* at *m/e* values of 259 and 241 showed the presence of a 14α-methyl group. Sterol P is therefore 24-methylene-lanost-8-en-3β-ol and is the sterol present in zone *Y* of the argentation TLC.

Fatty acid moieties of the sterol esters, triglycerides and phospholipids

The lipid from a batch of mycelium identical to that used in the sterol analyses was chromatographed on a 150 g alumina column and the following fractions collected, (1) 2% E/P, (2) 8% E/P, (3) 20% E/P, (4) MeOH. Fraction 3, which contained the unesterified sterols, was discarded. The sterol esters, present in Fraction 1 were purified by TLC (system 2) and saponified; the resulting fatty acids were extracted. Fraction 2 was subjected to preparative TLC (system 2) and the large triglyceride zone, co-chromatographing with marker tripalmitin (R_f 0·28), was recovered. The triglyceride was then saponified and the resulting fatty acids extracted.

A TLC examination (system 3) of fraction 4 revealed the presence of several phospholipid zones (R_fs 0·05, 0·10, 0·13, 0·30, 0·34, 0·40, 0·57, 0·63 and 0·84) staining with the molybdenum blue reagent.[30] The residue of the mycelium remaining after Me_2CO–Et_2O extraction was re-extracted several times with MeOH; the material extracted in this way produced an identical pattern of phospholipid zones when subjected to TLC (system 3). The major zone (R_f 0·56) co-chromatographed with egg lecithin; the other main zones had R_f values of 0·30 and 0·84. Fraction 4 and the MeOH extract were saponified separately and the fatty acids extracted.

The fatty acids derived from the sterol esters, triglycerides and the two phospholipid fractions were then analysed as their methyl esters by GLC. The identity of the fatty acid methyl esters was established by cochromatography with standards and by hydrogenation followed by re-chromatography.

The results of the fatty acid analysis are shown in Table 2.

DISCUSSION

The sterol components of the sterol esters of *P. blakesleeanus* have been shown to include ergosterol, episterol, ergosta-7-en-3β-ol, ergosta-7,22-dien-3β-ol, cholesterol, lanosterol, 24-methylene-lanost-8-en-3β-ol and traces of what is probably 4α-methyl-ergosta-

[29] GOAD, L. J. and GOODWIN, T. W. (1967) *European J. Biochem.* **1**, 357.
[30] VASKOVSKY, V. E. and KOSTETSKY, E. Y. (1968) *J. Lipid Res.* **9**, 396.

TABLE 2. COMPOSITION OF THE FATTY ACIDS DERIVED FROM THE STEROL ESTERS, TRIGLYCERIDES AND PHOSPHOLIPIDS OF *Phycomyces blakesleeanus*

Fatty acid[1]	Percentage composition[2] Sterol esters	Triglycerides	Phospholipid[3]	Phospholipid[4]
12:0	0·0	0·1	0·2	0·2
12:1	0·0	0·1	0·2	0·2
13:0	0·0	0·0	0·0	0·0
13:1	0·0	0·0	0·0	0·0
14:0	1·9	1·2	1·0	0·4
14:1	0·4	0·6	0·8	0·5
15:0	0·8	0·4	0·6	0·4
16:0	13·7	32·0	26·4	27·3
16:1	4·1	5·3	6·2	4·3
16:2	0·0	0·7	0·0	0·3
17:0	1·1	0·3	0·3	0·4
17:1	0·5	0·3	0·4	1·1
18:0	5·5	8·4	4·2	2·7
18:1	37·9	28·1	31·0	30·3
18:2	13·6	11·2	13·9	16·6
18:3(γ)*	14·2	8·9	12·1	14·4
19:1†	0·5	0·2	0·8	0·8
20:0	1·7	0·7	0·4	0·0
21:0	0·5	0·1	0·3	0·0
22:0	0·9	0·7	0·6	0·0
>22:0	2·5	1·0	0·0	0·0

* γ-Linolenic acid(octadeca-6,9,12-trienoic acid).

† This is a C_{19} unsaturated fatty acid, probably with one double bond.

The fatty acids were analysed by GLC of their methyl esters on 10% SP 1000.

[1] The number before the colon is the number of carbon atoms in the fatty acid; the number after the colon is the number of double bonds present.

[2] The composition is expressed as a percentage of the total fatty acids, using peak areas corrected for differences in FID response of the different fatty acids.

[3] Fraction 3, eluted with MeOH, from an alumina chromatography of the lipid extracted from *P. blakesleeanus* with Me_2CO and Et_2O.

[4] Fraction obtained after re-extraction with MeOH of the residue of the mycelium remaining after the Me_2CO–Et_2O extraction.

8,24(28)-dien-3-β-ol. The finding of cholesterol, admittedly in small quantities, was somewhat unexpected since McCorkindale *et al.*,[31] in a survey of the sterol composition of various species of Phycomycetes, had found a significant but not absolute correlation between sterol composition and cell wall composition. Phycomycete species of the orders Saprolegniales and Leptomitales, which have cellulose cell walls, synthesized mainly cholesterol, 24-methylenecholesterol, desmosterol and fucosterol whilst those of the order Mucorales, which has chitin cell walls, produce mainly ergosterol and 22-dihydroergosterol. *P. blakesleeanus*, being in the Mucorales and having chitin walls, would not be expected, on this basis to have cholesterol esters. However McCorkindale *et al.*[30] did find small amounts of unesterified cholesterol in two species of Mucorales, *Mucor hiemalis* and *Absidia glauca* and did not examine the sterol esters of the eight species of Mucorales they studied. More detailed examination of the sterols of fungi may show that cholesterol is more widespread than previously supposed, as was found in the case of the higher plants.

[31] MCCORKINDALE, N. J., HUTCHINSON, S. A., PURSEY, B. A., SCOTT, W. T. and WHEELER, R. (1969) *Phytochemistry* **8**, 861.

The main fatty acids found in the sterol esters were the C_{16} species, palmitic and palmitoleic acids and the C_{18} species, stearic, oleic, linoleic and γ-linolenic acids. These species have been shown to be the major fatty acids of the lipid of *P. blakesleeanus*.[32–34] Smaller quantities of other fatty acids of the range of C_{14}–C_{22} were present including some with odd numbers of carbon atoms. The same range of fatty acids was found in the triglycerides and phospholipids but the compositions were significantly different. This was particularly marked in the case of palmitic acid where the percentage present in the triglycerides and phospholipids was double that in the sterol esters. A similar situation occurs in *A. fumigatus* where the composition of the fatty acids esterified to sterols and polyprenols is quite different.[15] Random esterification is therefore not taking place in either organism. This would suggest that a particular range of sterol esters are required, pointing to particular structural or metabolic role in the organism.

EXPERIMENTAL

Organism and cultural conditions. Batches of 1·5 l. medium[18] were inoculated with 100 ml aliquots of a linear-phase culture of *Phycomyces blakesleeanus* Burgeff, (−) strain, and cultured on a gyrotary shaker for 60 hr at 24° under constant illumination (3750 lx).

Extraction of lipid. The mycelium was harvested by filtration through cheese-cloth, washed with H_2O and extracted exhaustively firstly with Me_2CO and then with Et_2O. The resulting lipid extract was then partitioned between Et_2O and H_2O. The Et_2O phase was washed free of Me_2CO, dried and evaporated to yield a red oil.

Column chromatography. Total lipid and lipid fractions were chromatographed on columns of acid washed, Brockmann Grade 3 alumina (Woelm) developed in a stepwise manner with increasing concentrations of dry, peroxide-free Et_2O in petrol. and finally, when phospholipids were required, with MeOH.

Thin layer chromatography. System 1: silica gel G (0·25 or 0·5 mm thick) impregnated with Rhodamine 6G [35] developed with $CHCl_3$. System 2: silica gel G (0·25 or 0·5 mm thick) impregnated with Rhodamine 6G developed with C_6H_6–petrol. (2:3, v/v). System 3: silica gel G (0·25 mm thick) developed with $CHCl_3$–MeOH–H_2O (65:24:4, by vol.). Argentation TLC: silica gel G (0·25 mm thick) impregnated with 10% (w/w) $AgNO_3$ developed with $CHCl_3$–Me_2CO (19:1, v/v).

Gas-liquid chromatography. Sterols were analysed on 1·8 m × 4 mm int. dia. glass columns packed with either 3% OV-1 on 100/120 mesh Gas Chrom Q or 5% QF-1 on 80/100 mesh Gas Chrom Q. The former column was operated isothermally at 240° and the latter at 235°. The carrier gas was argon flowing at 40 ml/min and detection was by FID. Cholestane was chromatographed with each sample and R_ts were determined relative to cholestane. GC-MS of sterol mixtures was carried out on a Pye 104 gas chromatograph fitted with a 1·5 m × 4 mm int. dia. glass column packed with 1% JXR on 100–120 mesh Gas Chrom Q at 250°, combined with an AEI MS-12 mass spectrometer. Fatty acid methyl esters were analysed on 1·8 m × 4 mm int. dia. glass columns packed with 10% SP 1000 on 100–120 mesh Chromosorb WAW. Dual column temp. programming was used; the initial temp. was 160° rising at 7·5°/min to a final temp. of 240° which was then held. The carrier gas was argon flowing at 40 ml/min and detection was by FID. The percentage composition of mixtures was estimated from peak areas; the latter were corrected for differences in FID response of different fatty acid methyl esters.

Saponification of sterol esters, triglycerides and phospholipids. These lipids were refluxed for 1 hr in 6% (w/v) KOH in 90% (v/v) EtOH containing 0·25% (w/v) pyrogallol as an antioxidant. After dilution with four vol. H_2O, the unsaponifiable lipid was removed by repeated extraction with Et_2O. This procedure isolated the sterols derived from saponification of the sterol esters. The saponification mixture was then acidified to pH 1 with HCl and re-extracted with Et_2O to obtain the fatty acids.

Preparation of fatty acid methyl esters. This was accomplished in the usual manner with the boron trichloride–MeOH reagent.

Hydrogenation of fatty acid methyl esters. This was carried out by passing H_2 gas for 1 hr through a soln of the fatty acid methyl esters in ethyl acetate in the presence of Adam's catalyst.

Acknowledgements—We are indebted to Professor T. W. Goodwin, F.R.S., Department of Biochemistry, University of Liverpool for allowing our sterol samples to be analysed on his GC–MS apparatus and to Dr. L. J. Goad for his help with the running of the samples.

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